Cold Spring Harbor Perspectives in Biology
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TGF-β Family Signaling in Tumor Suppression and Cancer Progression

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Transforming growth factor-β (TGF-β) induces a pleiotropic pathway that is modulated by the cellular context and its integration with other signaling pathways. In cancer, the pleiotropic reaction to TGF-β leads to a diverse and varied set of gene responses that range from cytostatic and apoptotic tumor-suppressive ones in early stage tumors, to proliferative, invasive, angiogenic, and oncogenic ones in advanced cancer. Here, we review the knowledge accumulated about the molecular mechanisms involved in the dual response to TGF-β in cancer, and how tumor cells evolve to evade the tumor-suppressive responses of this signaling pathway and then hijack the signal, converting it into an oncogenic factor. Only through the detailed study of this complexity can the suitability of the TGF-β pathway as a therapeutic target against cancer be evaluated.

One of the hallmarks of the transforming growth factor-β (TGF-β) pathway is its pleiotropic nature. Exerting a wide range of functions, TGF-β is a critical cytokine in embryogenesis and tissue homeostasis. TGF-β can induce a large and diverse set of responses, ranging from the induction of tissue growth and morphogenesis in the embryo to activation of cellular cytostatic and death processes in epithelial cells. The nature of the pleiotropic response to TGF-β is determined by the cellular context and the integration of the TGF-β pathway with other signaling cascades.

Paradoxically, and within the concept of TGF-β pleiotropic responses, this cytokine inhibits cell proliferation and stimulates differentiation in normal cells, thus acting as a tumor-suppressor factor (Roberts and Wakefield 2003; Bierie and Moses 2006). In contrast, in advanced cancer, it induces tumor progression and metastasis, thus serving as an oncogenic factor. Tumor cells escape the growth inhibitory effects of TGF-β by accumulating mutations in components of the TGF-β signaling cascade or by selectively impairing the antitumoral response. In the latter case, cancer cells hijack several TGF-β-initiated pathways to their benefit, turning TGF-β into an oncogenic factor that induces angiogenesis, invasion, immunosuppression, and self-renewal of cancer-initiating

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Editors: Rik Derynck and Kohei Miyazono
Additional Perspectives on The Biology of the TGF-β Family available at www.cshperspectives.org

Advanced Online Article. Cite this article as *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a022277

cells. Researchers have explored the mechanisms underlying this phenomenon, revealing the biological complexity arising from the integration of various layers of signaling mediators, cell type, and cell function specificity, as well as stromal contribution. Here, we review the knowledge of the mechanisms involved in the transformation of the TGF-β pathway from a tumor suppressor to a tumor promoter factor and discuss the implication of this process for understanding cancer biology and treatment (Fig. 1).

THE NATURE OF A PLEIOTROPIC PATHWAY

The signaling pathway that conveys TGF-β inputs from membrane receptors to cellular responses has started to be clarified (Fig. 2). TGF-β binds to receptors at the cell surface, forming a bi-dimeric receptor complex of the TGF-β type I receptor (TβRI, also known as ALK-5) and TGF-β type II receptor (TβRII) (Derynck and Zhang 2003; Shi and Massagué 2003), and causes activation of TGF-β receptor

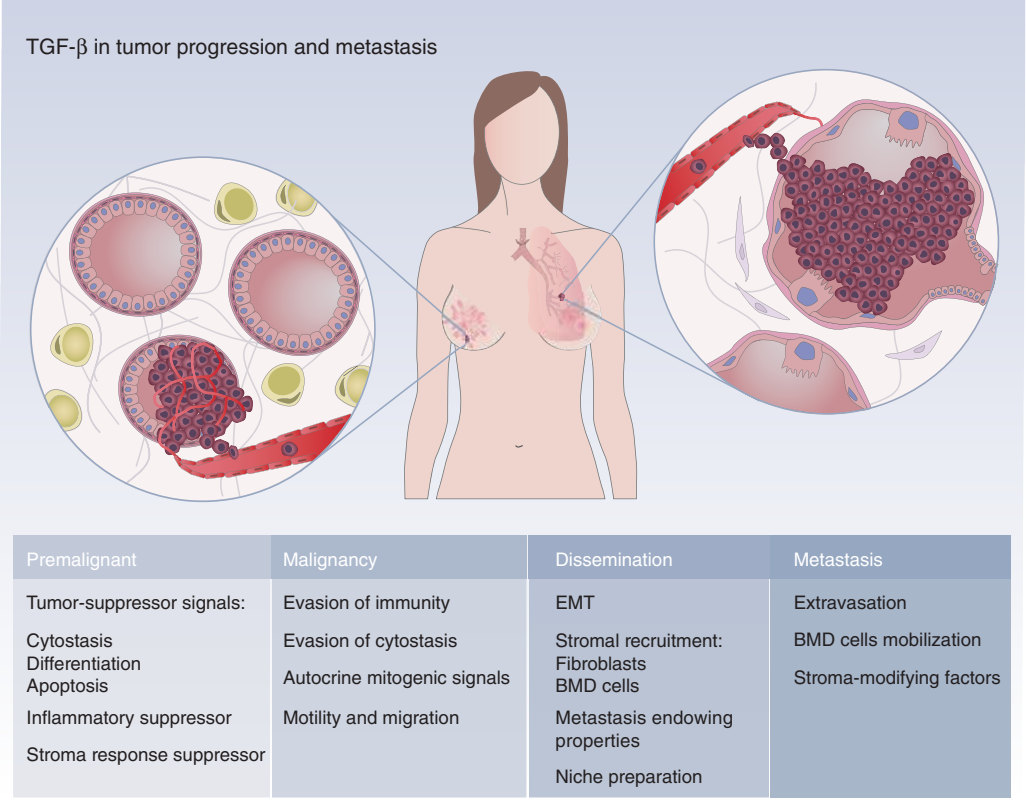

Figure 1. Transforming growth factor β (TGF-β) in tumor progression and metastasis. TGF-β limits the growth of normal epithelium and premalignant lesions. Loss of the cytostatic response to TGF-β can occur by mutations in or loss of TGF-β receptors, Smads, or by specific loss of mediators of the TGF-β cytostatic responses. In addition, tumors evade the immune response and increase autocrine mitogenic signals and motility and migration during malignancy. Tumor cells that have lost the cytostatic response may undergo epithelial-to-mesenchymal transition (EMT) in response to TGF-β and become more invasive. Concurrently, these cells may use TGF-β to evade the immunosuppressive environment and induce angiogenesis and systemic dissemination. Finally, adherence of tumor cells to the endothelium and/or extravasation of tumor cells at sites of metastasis, such as lung, can be enhanced by TGF-β signaling. Similarly, stroma-modifying factors, such as those that promote osteolytic bone metastasis by breast cancer cells, are driven by TGF-β signaling. BMD, bone marrow–derived.

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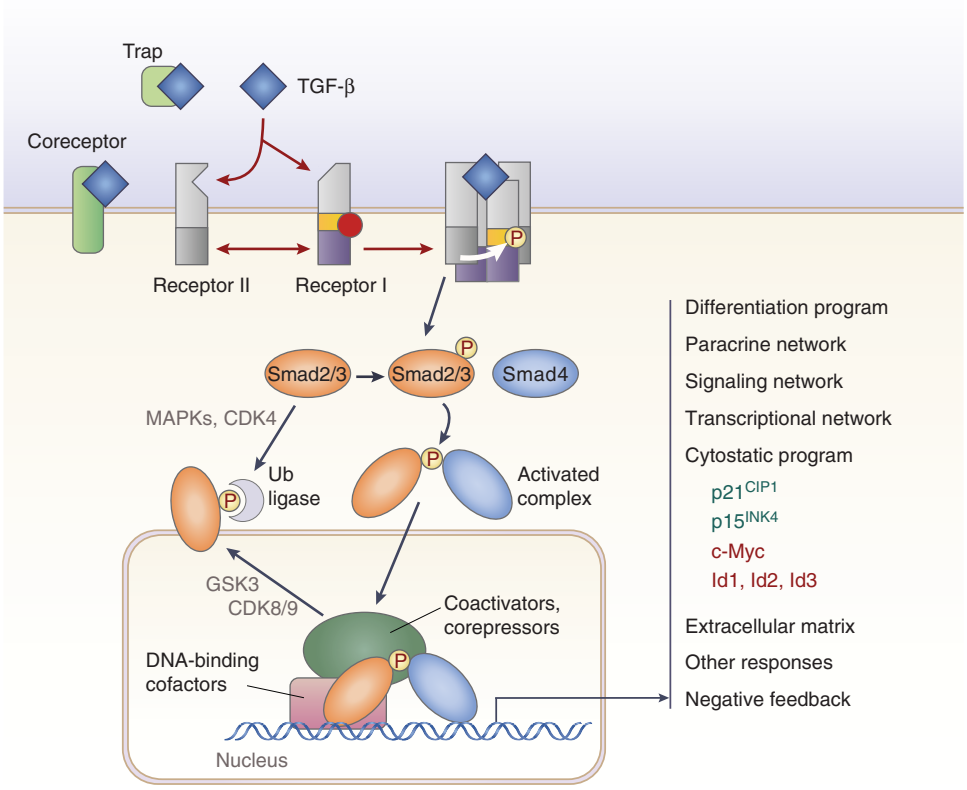

Figure 2. The transforming growth factor β (TGF-β)-induced Smad signaling pathway. TGF-β binds to the type II receptor and recruits the type I receptor, whereby the type II receptor phosphorylates and activates type I receptor. The type I receptor, in turn, phosphorylates receptor-activated Smads (Smad2 and Smad3) at the carboxy-terminal SXS motif, which results in release of these Smads from the receptor complex in the cytoplasm and triggers their translocation into the nucleus. Smad4 acts as a common partner of activated Smads to help execute their function. Smad proteins continuously undergo nucleocytoplasmic shuttling and interact with nuclear pore complexes. Once in the nucleus, activated Smad proteins form complexes that regulate target gene transcription, generating hundreds of early gene responses. Mechanisms of phosphorylation and polyubiquitylation account for the signal termination of the activated Smads. On the *right*, TGF-β target genes in epithelial cells are grouped on the basis of their biological responses. Highlighted in red are gene responses repressed by TGF-β, and in green are gene responses induced by TGF-β. These are central for the cytostatic program induced by TGF-β.

transmembrane dual specificity kinase (Attisano et al. 1992; Lin et al. 1992; Franzén et al. 1993; Tsuchida et al. 1993; Takumi et al. 1995; Luo and Lodish 1997). On ligand binding, the type II receptor phosphorylates serine and threonine residues in the type I receptor, which subsequently propagates the signal through Smad activation (Wrana et al. 1994). Phosphorylation switches this region from serving as a docking site for an inhibitor, FKBP12, to a docking site for its various substrates, including the Smad family of transcription factors (Huse et al. 1999, 2001). In the absence of phosphorylation, Smads are transcriptionally inert but undergo constant nucleocytoplasmic shuttling through the nuclear pore complex (Xu et al. 2003; Chen et al. 2005a; Schmierer and Hill 2005; Varelas et al. 2008). Receptor-mediated phosphorylation of Smads occurs at their carboxy-terminal regions and induces the accumulation of receptor-activated Smad (R-Smad) proteins in the nucleus (Hoodless et al. 1996; Liu et al.

1996; Kretzschmar et al. 1997). The phosphorylated motif of the R-Smad generates a docking site for Smad4 (Wu et al. 2000, 2001), which is not a receptor substrate but an important component of the resulting R-Smad transcriptional complexes (Lagna et al. 1996; Shi and Massagué 2003).

Not all responses to TGF-β are Smad-dependent, thus further increasing the level of complexity. TGF-β has been shown to activate other mediators, such as the extracellular signal-regulated kinase (Erk), c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs), phosphatidylinosititol 3-kinase (PI3K), PP2A phosphatases, Rho family members, and many others (reviewed in Derynck and Zhang 2003; Derynck et al. 2014). These alternative pathways are activated by TGF-β in a cell-type-dependent manner, and their biochemical associations with the activated receptors are variable. Not surprisingly, the expansion of TGF-β signaling beyond the canonical pathway implies yet another layer of diversity and thus supports the pleiotropic responses. TGF-β-induced epithelial-to-mesenchymal transition (EMT) involves Par6 activation and ubiquitin-mediated degradation of RhoA at tight junctions (Ozdamar et al. 2005), as well as TGF-β-induced Akt activation, thus causing mTOR activation and enhanced protein synthesis (Lamouille et al. 2012; Fruman and Rommel 2014). Additionally, ShcA phosphorylation by the TβRI receptor causes activation of Erk1/2 MAPK signaling, whereas activation of p38 MAPK and/or JNK are caused by the recruitment of the E3 ubiquitin ligases TRAF4 or TRAF6 to the TGF-β receptor complex, which, in turn, causes TAK1 kinase activation (Sorrentino et al. 2008). These inputs may directly regulate the stability and activities of Smads (Zhang 2009; Mu et al. 2012). Thus, these signals instruct nontranscription changes and also cooperate with Smad-mediated gene expression, tuning the outcome of the TGF-β signal (Derynck et al. 2014).

R-Smad activation typically leads to the formation of a complex with Smad4 and hundreds of immediate gene activation or repression responses (Feng et al. 1998; Zhang et al. 1998). Smad proteins consist of a Mad homology (MH) 1 and an MH2 domain connected by a linker region (Derynck and Zhang 2003). The former is mainly responsible for DNA binding (Shi et al. 1998), whereas the latter establishes contacts with anchors for cytoplasmic retention (Wu et al. 2000), receptors for activation (Huse et al. 2001), and nucleoporins for nucleocytoplasmic translocation (Xu et al. 2002), and both domains partner Smads and other nuclear transcription factors for the assembly of transcriptional complexes (Wu et al. 2001). R-Smad–Smad4 complexes have DNA-binding activity; however, they must associate with other cofactors to achieve DNA interaction with high affinity and selectivity. To date, many partners have been reported among transcription factor families, including the AP1, forkhead, basic helix-loop-helix (bHLH), and zinc finger transcription factors (Feng and Derynck 2005). Each complex is tailored for a particular regulatory DNA-binding region and is thus responsible for the expression of a given set of genes. Activated Smad complexes also mobilize coactivators and corepressors, as well as chromatin remodeling factors. Collectively, permutations and variations of these various players explain how and why a single TGF-β stimulus can activate or repress several genes at a time.

Gene response termination is also regulated at the level of Smad complexes (Fig. 2). It has been proposed that PPM1A protein phosphatase terminates R-Smad carboxy-terminal phosphorylation, thus stimulating the rapid exclusion of activated Smads from the nuclei (Lin et al. 2006). There is also evidence that R-Smads undergo selective ubiquitin-mediated degradation (Lo and Massagué 1999). The involvement of Smad ubiquitylation regulatory factors (Smurfs) in this process and the manner in which phosphorylated R-Smads are recognized for degradation have become clearer. Smads are phosphorylated in the linker region by cyclin-dependent kinase (CDK) 8 and CDK9 (Alarcón et al. 2009). This event attracts factors that support the transcriptional function and/or the phosphorylation of the linker region by glycogen synthase kinase 3 (GSK3) (Fuentealba et al. 2007; Sapkota et al. 2007). These resulting new

binding sites support the recruitment of Smurf E3 ubiquitin ligase or NEDD4L, which target Smad proteins for degradation through polyubiquitylation and proteasome degradation (Gao et al. 2009). Similarly, SCP1-3 protein phosphatases may also account for Smad1 carboxy-terminal and linker region dephosphorylation (Sapkota et al. 2006). Cellular stress, growth factors, inflammatory cytokines, and other stimuli, mediated by MAPK signaling pathways, are responsible for the phosphorylation of the linker region. This event is proposed, among other functions, to expose the binding site of Smurf1 E3 ubiquitin ligase, as modeled in Smad1 (Sapkota et al. 2007). Similarly, the phosphorylation of the linker regions of Smad2 and Smad3 may enhance degradation by other ubiquitin ligases (Aragón et al. 2011; Macias et al. 2015).

Furthermore, TGF-β signaling is negatively controlled by a feedback loop mediated by the inhibitory Smads—namely, Smad6 and Smad7. Smad6 and Smad4 compete for Smad1 binding, whereas Smad7 and Smad6 recruit Smurf to inactivate signaling at the TGF-β and bone morphogenetic protein (BMP) receptor level (Goto et al. 2007). Smurf-mediated polyubiquitylation targets the receptors for degradation by the proteasome. Interestingly, the deubiquitylating enzymes USP15 and USP4 have been described to counteract the function of Smurfs and to promote receptor stabilization (Eichhorn et al. 2012; Zhang et al. 2012). The physiological levels of TGF-β receptors are determined by the balance between Smurfs and USP15 and/or USP4. The transcriptional repressors c-Ski and SnoN (Ski-like) can also directly inhibit the transcriptional function of Smads (Luo et al. 1999; Stroschein et al. 1999; Zhu et al. 2007). Additionally, other transcriptional repressors encoded by chimeric genes (AML1/EVI-1 t(3,21) or AML1/ETO t(8,21)) interact with Smad3 (Letterio 2005). Beyond the main components of the Smad signaling pathway, several other actors have been identified that stringently regulate the activity of each step of this powerful signaling process (for review, see Massagué 2012).

Given the large set of transcription factors that interact with Smads and the broad set of genes regulated, the TGF-β response is pleiotropic, showing various levels of coordination and subjected to context dependence. The various structural Smad motifs facilitate interactions with a wide spectrum of partners. Analogously, each complex arrangement shares specific enhancer element configurations at the DNA-binding level. Thus, within the magnitude of the TGF-β response, these common transcriptional regulatory elements define synexpression groups of genes that are coordinately regulated (Niehrs and Pollet 1999; Gomis et al. 2006a). Finally, distinct cell types and contexts define the availability of Smad partners, thus limiting the TGF-β response to specific cellular scenarios. This operating procedure supports the ample pleiotropic responsiveness of TGF-β and highlights the devastating consequences of its misuse in cancer.

THE TUMOR-SUPPRESSIVE RESPONSE OF TGF-β

TGF-β has a crucial role in tissue homeostasis. In particular, in normal epithelial cells, this growth factor can induce a potent antiproliferative response and promote cell differentiation and apoptosis. These responses make TGF-β a tumor-suppressive factor in early stage tumors. Substantial attention has been directed to the molecular mechanisms involved in the anticoncogenic effect of TGF-β.

The Cytostatic Program

TGF-β inhibits the progression of cell-cycle phase G_1 through two sets of events, namely, the induction of expression of CDK inhibitors and the suppression of c-Myc expression (Fig. 3). In epithelial cells, TGF-β induces the expression of the CDK inhibitor $p15^{INK4b}$, which inhibits the formation of cyclin D complexes with CDK4 or CDK6, and of $p21^{CIP1}$, which inhibits the formation of cyclin E or cyclin A complexes with CDK2. The Smad3–Smad4 complexes associate with FoxO transcription factors to target the promoters of the *CDKN2B* gene, which encodes $p15^{INK4b}$, and of *CDKN1A*, which encodes $p21^{CIP1}$, for transcriptional activation

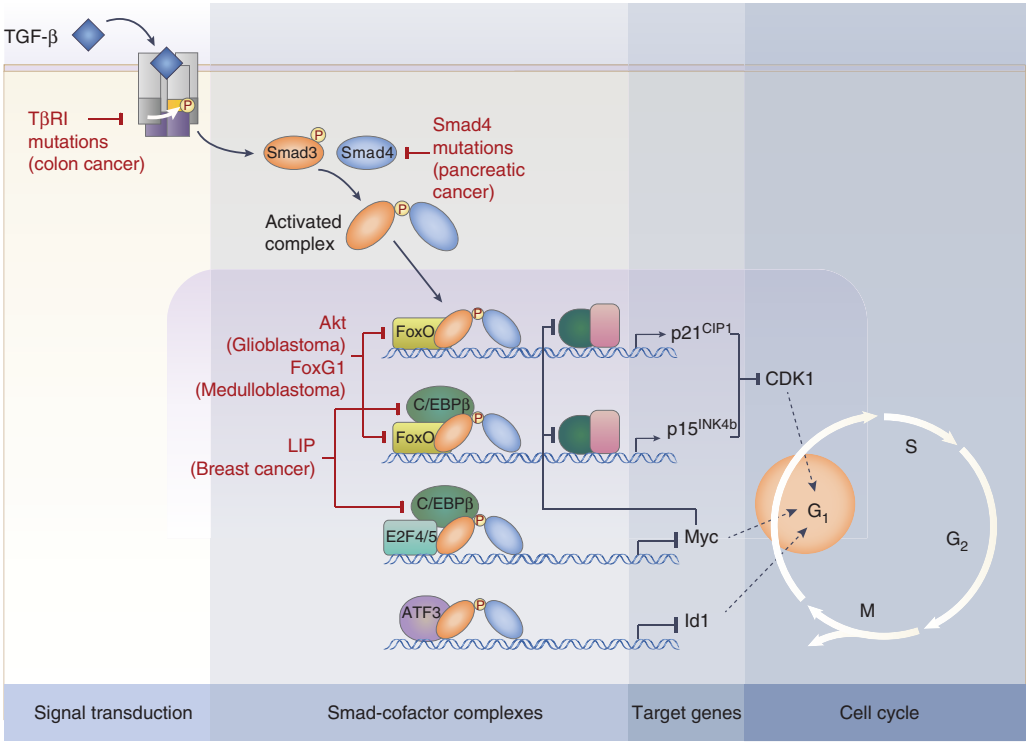

Figure 3. The transforming growth factor β (TGF-β)-induced transcriptional program and its alterations in cancer. The diagram depicts mutations or alterations that occur in genes that encode mediators of the TGF-β signaling pathway in distinct types of human cancers. Shown are the transcriptional components underlying the principal TGF-β cytostatic responses in epithelial cells. Indicated in red are the targets of alterations present in distinct types of human cancers converging on the TGF-β target genes that mediate cell cycle arrest.

(Seoane et al. 2001, 2004; Gomis et al. 2006a). The induction of these genes also requires the transcription factor Sp1 (Pardali et al. 2000). Another CDK inhibitor, $p27^{Kip1}$, is mobilized from an inactive state bound to cyclin D-CDK4 to an active state that is displaced from these complexes by $p15^{INK4b}$ to target the complexes of cyclin E or cyclin A with CDK2. TGF-β stimulates the expression of $p21^{CIP1}$ in T cells (Wolfraim et al. 2004), $p57^{Kip2}$ in hematopoietic stem/progenitor cells (Scandura et al. 2004), and $p15^{INK4b}$ and $p21^{CIP1}$ in astrocytes and neural progenitor cells (Seoane et al. 2004). Thus, the particular CDK inhibitors involved in the cytostatic response to TGF-β depend on the cell type and context.

c-Myc is a key transcriptional inducer of cell growth and division. In keratinocytes and mammary epithelial cells, down-regulation of *Myc* expression is mediated by a TGF-β-induced protein complex containing the Smad3–Smad4 complex, and the transcription factors p107, E2F4 or E2F5, and CCAAT/enhancer binding protein β (C/EBPβ) (Chen et al. 2002; Gomis et al. 2006b). The complex of Smad3 and Smad4 with E2F4 or E2F5 recognizes a proximal element in the *Myc* promoter, and p107 is thought to recruit corepressors. Interestingly, C/EBPβ is required for the repression of *Myc* expression by this complex and for activation of $p15^{INK4b}$ expression by a Smad3–Smad4–FoxO complex (Gomis et al. 2006a). Thus, C/EBPβ coordinates the responses of the genes encoding $p15^{INK4b}$ and c-Myc to TGF-β. Additional coordination is provided by the transcription factor Myc-interacting Zn finger protein-1 (Miz-1), which, in proliferating cells, recruits c-Myc as a repressor to the transcriptional start regions of the

CDKN2B and *CDKN1A* promoters (Seoane et al. 2001, 2002; Staller et al. 2001). As cotransducers of Smad signals, FoxO, E2F4 or E2F5, and C/EBPβ integrate multiple inputs into the TGF-β cytostatic program.

Smad-independent pathways downstream from TGF-β have been also implicated in the antiproliferative response to TGF-β (Derynck and Zhang 2003). For example, TGF-β induces the dephosphorylation of $p70^{S6K}$ by PP2A, thus leading to cell cycle arrest (Petritsch et al. 2000).

Effects on Cell Differentiation

In some cases, TGF-β and other members of its family may influence cell differentiation. Through Smad-mediated transcriptional repression or activation of various genes, TGF-β signaling may cause changes in cellular differentiation. TGF-β promotes the differentiation of mesenchymal precursors into fibroblasts and myofibroblasts at the expense of adipocyte, myocyte, and osteoblast differentiation fates (Derynck and Akhurst 2007). TGF-β also regulates differentiation by controlling the expression of Id proteins (inhibitor of differentiation/DNA binding), which inhibit some differentiation pathways by interfering with pro-differentiation bHLH transcription factors (Ruzinova and Benezra 2003). In epithelial and endothelial cells in culture, BMP stimulates *Id1* expression and TGF-β represses it (Korchynskyi and ten Dijke 2002; Kang et al. 2003a). BMP-induced binding of Smad1 to the *Id1* promoter supports transcriptional activation, whereas TGF-β signaling through Smad3 induces the expression of the repressor activating transcription factor 3 (ATF3), which is then recruited by Smad3 to the *Id1* promoter and represses *Id1* expression (Kang et al. 2003a). Id1 enhances Ras-driven mammary tumorigenesis in mice by bypassing senescence (Swarbrick et al. 2008). In a xenograft model using a Ras-transformed human breast epithelial cell line, TGF-β down-regulates *Id1*, thereby suppressing tumor formation by these cells and imposing a less proliferative phenotype (Tang et al. 2007). These findings suggest that *Id1* repression mediates cell differentiation as a tumor-suppressive response to TGF-β.

Induction of Apoptosis

In addition to the regulation of the cell cycle and cell differentiation, TGF-β can trigger apoptosis. Mechanisms of TGF-β-induced apoptosis include an increase in the expression of death-associated protein kinase DAPK in hepatoma cells (Jang et al. 2002), the expression of the signaling factor GADD45β (growth arrest and DNA damage 45β) in hepatocytes (Takekawa et al. 2002), and the activation of death receptor FAS and binding of the proapoptotic effector Bim to Bcl-2 and Bcl-X_L in gastric carcinoma cell lines (Ohgushi et al. 2005). In addition, Smad interactions with the p38 MAPK activator DAXX have also been proposed to mediate the proapoptotic effects of this growth factor (Perlman et al. 2001). TGF-β promotes apoptosis in hepatocytes and B lymphocytes through Smad3-dependent transcription of the gene encoding phosphatase MKP2, which enhances the proapoptotic effect of the Bcl-2 family member Bim (Ramesh et al. 2008). Interestingly, TGF-β inhibits the expression of prosurvival protein survivin, as well as the activity of Akt in colon cancer cells, thus leading to apoptosis (Wang et al. 2008). Moreover, it has been shown that the TGF-β–Smad pathway, in cooperation with the transcription factors Rb and E2F4, suppresses survivin expression in prostate epithelial cells (Yang et al. 2008).

In addition to the Smad-dependent responses, TGF-β can induce apoptosis by the TRAF6–TAK1–JNK/p38 pathway in some cell types. The E3 ligase TRAF6 was found to have a fundamental role in TGF-β-induced apoptosis (Sorrentino et al. 2008; Yamashita et al. 2008). TRAF6 binds constitutively to a consensus binding site in TβRI. Ligand-dependent oligomerization of the TβRII–TβRI complex leads to autoubiquitylation of TRAF6, and active TRAF6 subsequently causes polyubiquitylation of TAK1, which promotes its kinase activity. Activated TAK1 then phosphorylates and activates MKK3 or MKK6, which in turn activates p38 MAPK, thereby leading to apoptosis (Yamaguchi et al. 1995; Shibuya et al. 1996). Smad7 acts as a scaffolding protein to facilitate the activation of this MAP kinase cascade (Sor-

rentino et al. 2008; Yamashita et al. 2008). Some of the proapoptotic effects of TGF-β can be linked to the tumor suppressor p53 (Zhang et al. 2006), which in turn is regulated by p38 MAPK and Smads. Interestingly, in hepatocytes, TGF-β induces cell death through reactive oxygen species (ROS) production (Sanchez et al. 1996). TGF-β induces ROS production by repressing the expression of antioxidant genes or by activating the expression of NADPH oxidase (Nox) (Franklin et al. 2003; Herrera et al. 2004). TGF-β-induced ROS production can promote apoptosis through a mitochondrial-dependent pathway, at least in part through the modulation of various members of the Bcl-2 family (Ramjaun et al. 2007).

Tumor Suppression through Paracrine Signals

In addition to its direct growth-inhibitory effects on target cells, TGF-β can restrict epithelial cell proliferation and tumor formation by blocking the production of paracrine factors in stromal fibroblasts and inflammatory cells. The expression of a transgene encoding a dominant-negative TβRII receptor in the mammary stroma increases the lateral branching of adjacent mammary ducts. Mice with a targeted deletion of *Tgfbr2* in fibroblasts of the prostate and forestomach show hyperplasia of the adjacent epithelia with progression to prostatic intraepithelial neoplasia and gastric squamous carcinoma, respectively (Bhowmick et al. 2004). These effects are accompanied by elevated expression of hepatocyte growth factor (HGF) in the *Tgfbr2*-defective fibroblasts and activation of the HGF receptor c-Met in adjacent epithelial cells (Bhowmick et al. 2004). By constraining the expression of mitogenic factors in stromal fibroblasts, TGF-β limits the paracrine stimulation of epithelial proliferation and suppresses tumor development.

ESCAPING THE TUMOR-SUPPRESSIVE EFFECT OF TGF-β

During tumor progression, tumor cells tend to escape the tumor-suppressive responses to TGF-β in the same way as they evade the action of other tumor suppressors, such as the p53 pathway. The evasion of cytostasis and other TGF-β-related homeostatic functions confers a strong selective advantage in malignancies (Seoane 2006). In this regard, tumor cells elude the tumor-suppressive effect of TGF-β through various mechanisms that have come to light.

In some cases, tumor cells acquire somatic mutations or epigenetic silencing in genes encoding components of the TGF-β–Smad signal transduction pathway (Smads and TGF-β receptors) so that they escape the antitumoral function of this cytokine (Fig. 3). In many other tumors, the components of the TGF-β signal transduction pathway are not affected, but cells become specifically resistant to the antiproliferative response to TGF-β (Fig. 3). Mutational inactivation of genes encoding core pathway components occurs in large subsets of colorectal, pancreatic, ovarian, gastric, and head and neck carcinomas. However, in breast and prostate cancers, gliomas, melanomas, and hematopoietic neoplasias, the cytostatic program of this cytokine is selectively impaired.

Genetic and Epigenetic Alterations in Components of the TGF-β Pathway

The Receptor Complex

Colon cancers with microsatellite instability (MSI) consistently accumulate replication errors in the TβRII gene *TGFBR2*. This pathological condition is caused by mutations in genes involved in the replication mismatch repair machinery, and associated with high CpG island methylation phenotype (Markowitz et al. 1995). Insertion or deletion of adenines in a 10-bp polyadenine track in the TβRII coding sequence are common in gastric, colorectal, biliary, and lung adenocarcinomas, causing the expression of a truncated, inactive receptor (Markowitz et al. 1995; Ogino et al. 2007; Shima et al. 2011). When these alterations occur, the second *TGFBR2* allele is subsequently inactivated (Massagué 2008). Analogous alterations in the *TGFBR2* gene occur in gastric tumors and gliomas (Izumoto et al. 1997) and in a fraction of microsatellite-stable colorectal tumors with

inherited mutations in mismatch repair genes. In the latter case, the mutations generally affect the TβRII kinase domain (Grady et al. 1999). Interestingly, breast and endometrial tumors with MSI do not accumulate *TGFBR2* mutations. Frameshift, missense, or hypomorphic mutations in the *TGFBR1* gene are uncommon, but occur in ovarian, breast, esophageal, and pancreatic cancers (Chen et al. 1998; Goggins et al. 1998; Wang et al. 2000).

Epigenetic alterations may also account for alterations in receptor expression. Decreased expression of TβRII or TβRI has been reported in lung, gastric, and prostate cancer, among others. For example, in gastrointestinal tumors, the methylation status of the *TGFBR1* promoter has been directly linked to its decreased expression. Hereditary mutations in the *BMPR1A* gene encoding the BMP type I receptor BMPRIA (ALK-3) cause juvenile polyposis, an autosomal dominant genetic disorder that predisposes patients to intestinal polyposis and cancer (Massagué et al. 2000; Derynck et al. 2001; Howe et al. 2001; Roberts and Wakefield 2003).

Analogously, genetic alterations that cause alterations in the expression of TGF-β coreceptors can also blunt the pathway signaling activity. For example, inherited mutations in *ENG* gene encoding the betaglycan-related protein endoglin, a well-established BMP-9 coreceptor, cause hemorrhagic telangiectasia syndrome and are also associated with early onset of juvenile polyposis syndrome (Gallione et al. 2010). Additionally, overexpression of proteins that trap TGF-β family members, including BMPs and activins, have also largely been associated with cancer progression and metastasis. Follistatin has been implicated in hepatocarcinogenesis (Rodgarkia-Dara et al. 2006), whereas Noggin and Gremlin have been reported to contribute to breast cancer bone and lung metastasis (Gao et al. 2012; Tarragona et al. 2012), as well as in skin basal carcinoma (Sneddon et al. 2006). Collectively, genetic inactivation of TGF-β core components results in the elimination of most or all TGF-β responses, including tumor-suppressor activities, and may result in outcomes that are very different from those due genetic alterations in effectors that only blunt part of the cytokine responses.

Signaling Mediators

Mutations in genes encoding R-Smads are rare in cancer and have been described in only a limited number of cases (Sjöblom et al. 2006). Specifically, mutations or loss of expression of *SMAD3* are infrequent. Loss of *SMAD3* expression has been reported in gastric cancer and T-cell lymphoblastic leukemia (Levy and Hill 2006), whereas loss of heterozygosity of the 18q21 chromosomal region containing *SMAD2* and *SMAD4/DPC4* (*deleted in pancreatic carcinoma, locus 4*) is observed in pancreatic and colon cancer (Massagué 2008).

Alterations of *SMAD4/DPC4* are frequently observed in cancer and considered mostly a late event in tumor progression. In pancreatic tumors, chromosome 18q21 deletions, commonly affecting *SMAD4*, and systematic mutations or chromosomal aberrations that affect the other allele are common. The estimated frequencies of *SMAD4* mutations in pancreatic cancers are within the range of mutations of other pancreatic oncogenes or tumor suppressors, such as *KRAS*, *TP53*, and *CDKN2A (INK4)* (Jaffee et al. 2002). In colorectal tumors without MSI, mutations in *SMAD4* are also recurrent. Esophageal and other cancers also show mutations in *SMAD4*, with variable penetration rates (Barrett et al. 1996; Lei et al. 1996). *Smad4* heterozygote inactivation in the context of the $Apc^{\Delta 716}$ mice, which serve a model for human familial adenomatous polyposis, promotes the development of polyps that are more malignant than those in $Apc^{\Delta 716}$ heterozygous alone. These show extensive stromal cell proliferation and submucosal invasion and support the notion that mutations in *SMAD4* play a significant role in the malignant progression of colorectal tumors (Takaku et al. 1998). The critical role of *SMAD4* inactivation in cancer progression was subsequently confirmed in pancreatic cancer (Bardeesy et al. 2006), despite the differential requirements of SMAD4 for each tissue in colon and pancreas development (Takaku et al. 1998; Bardeesy et al. 2006). Finally, germline

mutations in *SMAD4* have been described in a subset of juvenile patients with polyposis syndrome (Schwenter et al. 2012). This autosomal dominant inherited condition is characterized by the development of multiple hamartomatous tumors in the gastrointestinal tract and makes patients prone to cancer. Animal models provide insights into the physiological alterations and suggest a role for Smad4 beyond the mucosa epithelium. Genetically engineered mice models in which *Smad4* is specifically deleted in T cells or epithelia develop juvenile polyposis syndrome (Kim et al. 2006), suggesting that TGF-β signaling in various cells types is a causal effector of genetic syndromes that predispose to cancer.

The disruption of the TGF-β pathway can also occur at the level of mediators that repress the activity in response to antagonistic signals or feedback loops (Massagué 2008). Alterations that cause persistent high levels of Smad7 blunt TGF-β signaling and have been described in endometrial carcinomas and thyroid follicular tumors (Cerutti et al. 2003; Dowdy et al. 2005). Similarly, increased Smad6 expression also attenuates TGF-β family signaling, preventing its tumor-suppression function in pancreatic or breast cancer (Kleeff et al. 1999; de Boeck et al. 2016). As reported above for *Smad4* depletion in genetic models, Smad7 overexpression in immune cells has been associated with chronic inflammation in the colonic mucosa. The expression of Smad7 variants is linked to predisposition to cancer, as shown by GWAS studies (Broderick et al. 2007), and to hepatic metastasis in colorectal cancer (Halder et al. 2008). Other mechanisms of direct inhibition of Smad activity have been described in pathology. c-Ski and SnoN are transcriptional corepressors of Smad transcriptional function. Deletions and amplifications of both *SKI* and *SKIL*, the gene encoding SnoN, have been detected in gastrointestinal tumors (Zhu et al. 2007). Finally, genomic translocations also perturb the pathway at the level of transcriptional complexes. In acute myelogenous leukemia (AML), proteins encoded by chimeric genes resulting from genomic translocations, including AML1/EVI-1 t(3:21) and AML1/ETO t(8:21), are known to bind to Smad3 and suppress TGF-β signaling at the transcriptional level (Letterio 2005).

Selective Failure of the TGF-β Antitumor Response

Smad cofactors are among the key mediators of the pleiotropic TGF-β response. These are transcription factors that bind Smad complexes and facilitate their binding to specific gene promoters. In the case of cytostatic gene responses, $p15^{INK4b}$ and $p21^{CIP1}$, the transcription factors FoxO, C/EBP, and Miz-1 have been characterized as Smad cofactors. FoxO facilitates the binding of the Smad complex to the *CDKN1A* promoter, whereas C/EBP and Miz perform a similar role in the context of the *CDKN2B* promoter. FoxO is, in turn, regulated by other signaling cascades, such as the PI3K–Akt pathway. A highly active PI3K–Akt pathway prevents FoxO nuclear localization, which, at the same time, impedes the formation of a nuclear FoxO–Smad complex. Hence, in tumors with a highly active PI3K–Akt pathway, the TGF-β-induced $p21^{CIP1}$ expression is impaired, thus blocking the TGF-β cytostatic response (Seoane et al. 2004). In neuroepithelial cells, the corepressor FoxG1 binds FoxO, again impairing the induction of $p21^{CIP1}$ expression by TGF-β. FoxG1 is a transcription factor required for the development of the telencephalon in embryogenesis; however, it is highly expressed in glioblastoma, where it binds to the FoxO–Smad complex to recruit transcriptional corepressors such as Groucho, and prevent the induction of $p21^{CIP1}$ expression by TGF-β (Seoane et al. 2004). Hence, in glioblastoma, the expression of high levels of FoxG1 impairs the $p21^{CIP1}$ response, thus precluding the antiproliferative effect to TGF-β.

On the other hand, the c-Myc binding factor Miz-1 binds to the transcription initiator region of the *CDKN2B* and *CDKN1A* promoters by forming a complex with c-Myc, which acts as transcriptional repressor. $p15^{INK4b}$ expression is not induced by TGF-β in tumors overexpressing Myc, because a repressive Myc-Miz-1 complex localizes close to the transcription initiation sites of the *CDKN2B* and

CDKN1A promoters, thus impairing the TGF-β cytostatic program (Seoane et al. 2001, 2002).

Breast cancer cells from pleural fluids of patients with metastatic disease show normal TGF-β receptor expression and Smad functions, even though their cytostatic response to TGF-β is partially or completely lost. Half of the samples in this study lacked induction of $p15^{INK4b}$ expression and repression of c-Myc expression in response to TGF-β, despite retaining other TGF-β responses. This defect was found to be associated with overexpression of the dominant-negative C/EBPβ isoform LIP, which binds and inhibits the transcriptional active isoform LAP (Gomis et al. 2006b; Arnal-Estapé et al. 2010). Other studies establish an association between a high LIP:LAP ratio and tumor aggressiveness in breast cancer (Zahnow et al. 1997). Patient-derived metastatic breast cancer cells are also uniformly aberrant in the *Id1* response to TGF-β, which is induced instead of repressed (Padua et al. 2008). *Id1* expression is part of a lung metastasis gene expression signature associated with relapse in estrogen receptor-negative (ER^-) breast cancer patients (Minn et al. 2005). In xenograft assays in mice using human breast cancer cell lines, Id1 and Id3 are essential for tumor reinitiation after the cells enter the lung parenchyma (Gupta et al. 2007). Therefore, the *Id1* response to TGF-β in breast cancer switches from being tumor-suppressive to prometastatic.

Importantly, TGF-β induces the expression of Id1 in a specific population of glioblastoma cells that express high levels of CD44 and show cancer-initiating capabilities. Through the regulation of *Id1*, TGF-β induces the self-renewal of the $CD44^{high}/Id1^+$ cancer-initiating cells, thereby promoting tumor relapse in glioblastoma (Anido et al. 2010).

TGF-β SIGNALING IN CANCER PROGRESSION: TUMOR GROWTH, CELL MIGRATION, AND INVASION

Released from tumor-suppression constraints, cancer cells use the remaining TGF-β responses with impunity to support tumorigenic features, including cancer progression, which encompasses evasion of immune surveillance, tumor growth, migration, invasion, and metastasis.

Immune Evasion

TGF-β stands out as a physiological immunosuppressor in humans (Gold 1999), and, accordingly, mice that systemically lack TGF-β1 succumb to systemic inflammation and severe autoimmunity (Shull et al. 1992; Diebold et al. 1995). The effect of TGF-β on the immune system is pleiotropic, affecting both the adaptive and innate immune system, including the regulation of T cells, natural killer (NK) cells, and macrophages (Fig. 4). The immunosuppressive response to TGF-β allows tumors to evade the anticancer immune response, and, hence, TGF-β may be considered an appealing therapeutic target in the context of cancer immunomodulation (Li and Flavell 2008).

T Cells

TGF-β inhibits both the proliferation and activation of T cells, thereby suppressing the differentiation and function of this cell population (Ranges et al. 1987; Park et al. 1997; Ahmadzadeh and Rosenberg 2005; Zhang et al. 2005b). TGF-β has been shown to suppress pore-forming protein (PFP) expression in $CD8^+$ cytotoxic T cells and to prevent T-cell cytolytic activity (Smyth et al. 1991). Interestingly, the regulation of PFP expression and the cytotoxic potential is independent of the proliferative response to TGF-β (Smyth et al. 1991). In addition, TGF-β also inhibits the expression of granzyme A, granzyme B, perforin, Fas ligand, and interferon-γ, which together promote T-cell-mediated tumor cell cytotoxicity (Ahmadzadeh and Rosenberg 2005; Thomas and Massagué 2005). The expression of granzyme B and interferon-γ has been directly linked to Smad2 and/or Smad3 and ATF1 transcription factors downstream from the TGF-β signal (Thomas and Massagué 2005). TGF-β can inhibit $CD4^+$ T-cell proliferation through its effect on macrophages (Alleva et al. 1995). Although TGF-β is expressed in different cell populations, including the tumor cells (Rodón et al. 2014), and in

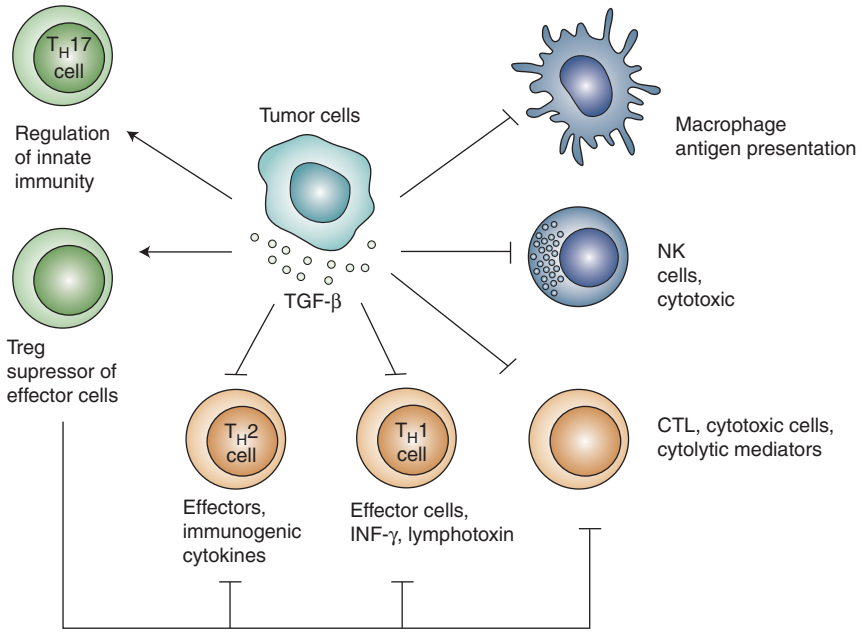

Figure 4. Pleiotropic effects of TGF-β on the immune system. TGF-β plays a role by controlling immune tolerance by the combined inhibition of most components of the innate (brown) and adaptive (blue) immune system directly or indirectly (green) through regulatory T cells. NK, Natural killer.

the tumor microenvironment (Chen et al. 2005b), regulatory T cells can secrete this cytokine, leading to the inhibition of $CD8^+$ T cells (Chen et al. 2005b). In addition, TGF-β can induce the differentiation of naïve $CD4^+$ T cells into regulatory T cells in murine peripheral blood and pancreatic cancer (Chen et al. 2003; Moo-Young et al. 2009). Importantly, systemic inhibition of TGF-β signaling has been shown to result in an antitumor response, in part mediated by the T-cell population (Kontani et al. 2006).

NK Cells

TGF-β inhibits NK-cell effector functions and in this way contributes to a permissive microenvironment for tumor progression. The proliferation of MDA-MB-231 mammary carcinomas and their metastatic dissemination are strongly reduced in response to TGF-β inhibition, and this effect is not observed in NK-cell-deficient beige nude mice (Arteaga et al. 1993). Interestingly, TGF-β regulates the effect of tamoxifen treatment in vivo through the regulation of NK-cell populations (Arteaga et al. 1999). In this context, the tamoxifen-resistant human breast cancer cell line LCC2 responds to tamoxifen on TGF-β neutralization. The antitumor effect of TGF-β inhibition was initially associated with the activity of TGF-β2 through the regulation of NK-cell-mediated cytotoxicity, because tumors grow irrespectively of TGF-β2 inhibition when cells are inoculated in mice that lack NK cells (Arteaga et al. 1999). This effect is attributed to the induction by TGF-β2 of a specific chemokine receptor repertoire, including the expression of CXCR4, and CXCR3, (Castriconi et al. 2013). Through these cytokines, TGF-β attenuates the expression of the NKp30 as well as the NKG2D receptors, which are required for NK-cell-mediated tumor cell death (Castriconi et al. 2003). Interestingly, inhibition of NKG2D-mediated NK-cell cytotoxicity by TGF-β enhances tumor growth and metastasis (Ghiringhelli et al. 2005; Smyth et al. 2006). Both NKG2D and NKp30 are recognition receptors that are triggered in response

to cellular stress—that is, viral infection, genomic stress, and are able to trigger cytotoxicity (Kruse et al. 2014; Carapito and Bahram 2015). Accordingly, an inverse correlation has been identified between TGF-β1 secretion and NKG2D expression in cancer patients (Lee et al. 2004), thereby indicating that the reduced NKG2D receptor expression may contribute to a decrease in NK-cell-associated tumor cell cytotoxicity (Lee et al. 2004). In addition, TGF-β has been shown to suppress MHC class I and MHC class II expression in a number of cell populations (Geiser et al. 1993; Ma and Niederkorn 1995; Lee et al. 1997; Gorelik and Flavell 2001). Importantly, the TGF-β-induced repression of MHC class I expression in tumor cells results in decreased NK-cell-mediated tumor cell death (Ma and Niederkorn 1995). Overall, increased TGF-β expression within the tumor microenvironment can lead to reduced NK-cell cytotoxic activity, thereby contributing to enhanced tumor progression and metastasis.

Macrophages

TGF-β promotes monocyte recruitment and macrophage differentiation (Li et al. 2006; Travis and Sheppard 2014). This cytokine has been shown to block both the priming of macrophages by interferon-γ and their activation by lipopolysaccharide, thus preventing these phagocytic cells from inducing tumor cell death (Haak-Frendscho et al. 1990). Many of the functional responses to TGF-β can be attributed to the regulation of gene expression in monocytes and macrophages. In monocytes, TGF-β promotes the expression of proinflammatory mediators, including interleukin-1 (IL-1) and interleukin-6 (IL-6), while suppressing oxygen-free radical production (Fontana et al. 1992). In macrophages, TGF-β suppresses the expression of chemokines including macrophage inflammatory protein 1α and 2 (MIP-1α, MIP-2) and the chemokine CXCL1 factor, the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukins IL-1β, IL-8, and IL-10 (McDonald et al. 1999), which in macrophages cause the acquisition of a deactivation state that prevents early, premature immune activation (Varol et al. 2015). Interestingly, targeted inactivation of *Eng* in cells of myeloid lineage, resulting in the absence of the TGF-β coreceptor endoglin, which controls monocyte-macrophage differentiation, results in phagocytic impairment and thus represses the contribution of macrophages to the initiation of the immune response (Ojeda-Fernández et al. 2016). TGF-β can also enhance the response to chemotactic signals that are known to be abundant within the tumor pro-inflammatory microenvironment, such as the stromal-cell-derived factor SDF-1/CXCL12 that acts through the CXCR4 receptor (Wang et al. 2001). In this regard, stimulation of monocytes and macrophages with the cytokine SDF-1 increases the expression of CXCR4 (Wang et al. 2001), leading to tumor progression (Orimo et al. 2005). Together, these results suggest that TGF-β regulates the recruitment, differentiation, activation, gene expression profile and response to external stimuli of macrophages, thereby directly affecting tumor progression.

Autocrine Mitogens

By disabling the cytostatic program of TGF-β, tumor cells turn TGF-β signaling to their advantage to promote cell proliferation, by stimulating the production of autocrine mitogenic factors. In advanced cancer, including glioblastoma, the TGF-β pathway acts as an oncogenic factor. On loss of the tumor-suppressor activity, including loss of induction of $p15^{INK4b}$ expression and/or inactivation of Rb, some tumors show aberrantly high TGF-β signaling. This activity can be sustained by an autocrine loop, whereby TGF-β induces the expression TGF-β2, leading to high levels of TGF-β2. Mechanistically, cAMP-responsive element binding protein 1 (CREB1) binds to the *TGFB2* promoter, and cooperates with Smad3 in TGF-β-induced activation of *TGFB2* transcription. The PI3K–Akt and ribosomal S6 kinase (RSK) pathways induce the phosphorylation of CREB1 that then binds the *TGFB2* promoter in complex with Smad3, generating the TGF-β2 autocrine loop in glioblastoma cells (Rodón et al. 2014). In certain cases, the hyperactivation of the

TGF-β pathway is not achieved by increased ligand expression but by stabilization of the TGF-β receptor complex. For example, the gene encoding the deubiquitylating enzyme USP15 that targets the TβRI receptor is amplified in some cancers, and this promotes the stabilization of the TβRI receptor, thus inducing increased activation of the TGF-β pathway (Eichhorn et al. 2012). Glioblastoma cells also produce platelet-derived growth factor B (PDGF-B) in response to TGF-β (Jennings and Pietenpol 1998) in a process that depends on the methylation state of the *PDGFB* gene (Bruna et al. 2007). Hypomethylation of the *PDGFB* promoter occurs in patients with high TGF-β expression and activated Smads and correlates with poor prognosis. Thus, the epigenetic state of the *PDGFB* gene contributes to the tumor cell fate in response to TGF-β.

Microenvironment as a Source of Mitogenic Signals

The loss of TGF-β-induced cytostasis in tumor cells allows the tumor to profoundly alter the host immune response to TGF-β. In parallel, the tumor cell can also adapt its environment to favor tumor initiation and progression. In this case, the process relies on the plasticity of both the tumor cells and stromal cells (Fig. 5). This process may be central in metastasis, as cross talk between disseminated tumor cells and distant organ microenvironments may establish tissue-specific dependence. The tumor microenvironment is comprised of various types of nonepithelial cell types, including fibroblasts, endothelial and immune cells, and extracellular matrix proteins (Fig. 5). The activation status of TGF-β signaling during tumor progression depends on whether the epithelial cells retain a functional TGF-β signaling pathway in full or in part, as described above (Massagué and Gomis 2006). However, in certain tumor types, such as colorectal and prostate cancers, elevated levels of TGF-β correlate with poor prognosis (Tsushima et al. 1996; Wikstrom et al. 1998) and relapse (Walker and Dearing 1992; Friedman et al. 1995; Calon et al. 2012), and are associated with increased TGF-β signaling in tumor-adjacent cells and not in the malignant epithelial tissue.

The mobilization of mesenchymal precursors and generation of myofibroblasts on TGF-β stimulation, as well as the recruitment of fibroblasts, are components of the protumorigenic and invasive contribution of the cytokine (De Wever and Mareel 2003). The myofibroblasts are highly motile, retain features of fibroblasts and smooth muscle cells, and facilitate tumor development as cancer-associated fibroblasts (De Wever and Mareel 2003; Allinen et al. 2004). These cells produce a range of proteases, cytokines (e.g., TGF-β, VEGF, EGF, PDGF, FGF, IGF-1, and type I collagen), and chemokines (e.g., CXCL12) that support cancer invasion, proliferation and angiogenesis. For example, in culture, these cells guide the invasion of colon cancer cells through a collagen matrix, in a process that depends on TGF-β. These and other observations support the notion that TGF-β signaling makes an important contribution to stroma- and cancer-associated fibroblasts (Hawinkels et al. 2009; Calon et al. 2012).

Fibroblasts in the tumor microenvironment differ from their normal counterparts, as can be seen by the expression of α-smooth muscle actin (α-SMA), fibroblast surface protein (FSP1, also known as S100A4), and fibroblast-activated protein (FAP) (Bauer et al. 2010; Navab et al. 2011). Interestingly, fibroblast-like cells both produce and respond to TGF-β, triggering a set of responses that support tumor development and progression. By means of genetic deletion, it has been shown that CLIC4 (chloride intracellular channel 4) is required for TGF-β-induced expression of α-SMA and extracellular matrix proteins in fibroblasts (Shukla et al. 2014). In addition, TGF-β triggers an autocrine signaling loop that sustains myofibroblast differentiation (Kojima et al. 2010). In addition to the recruited fibroblasts, cancer-associated fibroblasts were also shown to be derived from other cell types. Among these, TGF-β-induced transdifferentiation of endothelial cells into mesenchymal cells has been proposed to lead to expression of the fibroblast marker FSP1 and repression of expression of the endothelial marker CD31 (Zeisberg et al. 2007a). Addition-

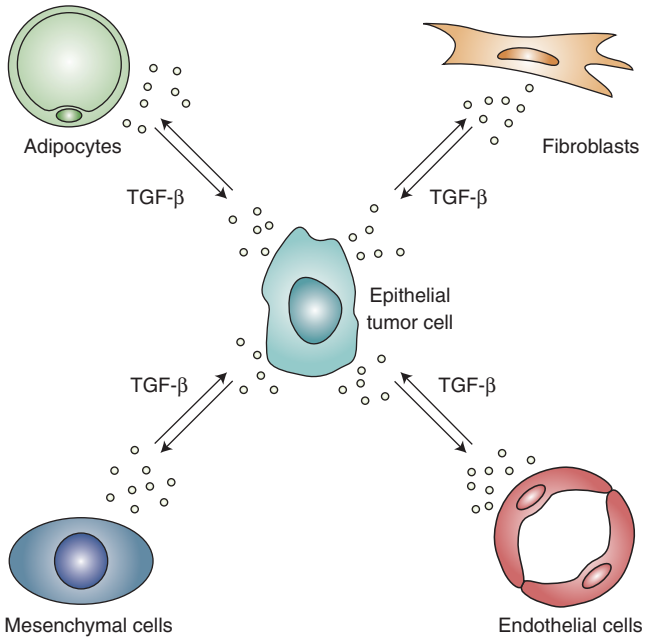

Figure 5. TGF-β signaling in cells adjacent to carcinoma cells and not in the malignant carcinoma cells. TGF-β is produced and activates signals in various cell types in the tumor environment. These include tumor epithelial cells, fibroblasts, endothelial cells, mesenchymal cells, and adipocytes.

ally, TGF-β-induced EMT was proposed to be a potential source of tumor fibroblasts (Oft et al. 2002; Petersen et al. 2003). Collectively, these lines of evidence point to a central role of TGF-β in the tumor microenvironment, where it supports the coevolution of the stroma, with epithelial transformation and progression toward malignancy.

Direct cell–cell and paracrine mechanisms mediate TGF-β cross talk between tumor epithelial cells and tumor-associated fibroblasts. In prostate cancer, tumor and stromal cells coevolve, and paracrine cytokines expressed by prostate fibroblasts, on loss of TGF-β responsiveness, further support tumor cell growth and dissemination (Bhowmick et al. 2004; Bhowmick and Moses 2005; Li et al. 2012). Similarly, breast cancers, melanomas, and gliomas that largely retain a functional TGF-β pathway and specifically escape the cytokine cytostatic effect, use TGF-β signaling to express prometastatic factors that then support growth at distant sites. These factors include JAGGED1, angiopoietin-like 4 (ANGPTL4), IL-11, and others described that are in the last section of this review.

A significant proportion of colon and pancreatic tumors have lost the TGF-β-induced cytostatic response as a result of alterations at the level of the receptors that inactivate the pathway (Markowitz et al. 1995). In colon cancer, tumor cells produce and release TGF-β in the microenvironment, thereby unveiling a prometastatic program that is associated with risk of relapse. This risk results from TGF-β activity on stromal cells, including fibroblasts, which increases the efficiency of dissemination, whereas treatment with TβRI inhibitors prevents metastasis (Calon et al. 2012, 2015). Central to the TGF-β stromal response is the secretion of IL-11 by cancer-associated fibroblasts, a process that triggers JAK-STAT3 signaling and confers a survival advantage to disseminated tumor cells (Calon et al. 2012, 2015). The TGF-β effects in the microenvironment are also observed in a mouse model of aggressive breast cancer metastasis, whereby cancer stem cells that are disseminated to the lungs misuse TGF-β signaling in the stroma to

create a niche that allows for metastasis (Malanchi et al. 2012). Interestingly, the TGF-β signaling-dependent education of the host stroma of the target organ by tumor cells is bimodal, whereby TGF-β signaling initially promotes EMT and niche activation, and the newly activated niche fibroblasts then promote a transition of the carcinoma cells toward a more epithelial phenotype to enable metastatic colonization (Del Pozo Martin et al. 2015). Similarly, low radiation triggers the education of the metastatic niche, dependent on TGF-β signaling (Biswas et al. 2007; Nguyen et al. 2011). These lines of evidence suggest that the dependence of the primary microenvironment on TGF-β could be exploited to improve the treatment of cancer.

Although there is clear agreement on the contribution of TGF-β to the tumor microenvironment, experiments using xenografts and genetically engineered mouse models report contra-intuitive effects of TGF-β signaling in fibroblasts. Strong evidence supports a role for TGF-β signaling in fibroblasts promoting tumor growth in breast, nonsmall cell lung and colorectal cancer (Kuperwasser et al. 2004; Navab et al. 2011; Nguyen et al. 2011; Calon et al. 2012, 2015; Malanchi et al. 2012). However, conditional inactivation of *Tgfbr2* in mouse fibroblasts has been shown to support prostate and forestomach tumorigenesis by impinging on HGF signaling through c-Met and Ron (Bhowmick et al. 2004; Cheng et al. 2008; Li et al. 2012). Indeed, in that scenario loss of TGF-β signaling in stromal fibroblasts may result in HGF-mediated cell cycle regulation in the tumor cells, by suppressing the expression of the CDK inhibitors, $p27^{Kip1}$ and $p21^{CIP1}$, and activating the expression of c-Myc (Bhowmick et al. 2004).

Beyond direct effects of TGF-β on the stroma and tumor cells, mechanisms that lead to an increase in active TGF-β level may also contribute to this process. TGF-β is secreted in complex with latent TGF-β binding protein (LTBP), which controls the latency of the cytokine. This represents another level of regulation, as mice deficient in LTBP–TGF-β association display increased inflammation and tumorigenesis (Shibahara et al. 2013). In addition, TGF-β signaling in fibroblasts is enhanced when these cells are combined with cancer cell-conditioned media. This observation points to a synergistic or additive effect of TGF-β with other secreted factors (Hawinkels et al. 2009). Increased expression of matrix metalloproteinase 9 and mechanical stress have been reported to enhance breast cancer malignancy as a result of increased TGF-β activation (Stuelten et al. 2005; Wipff et al. 2007). Similarly, senescence-associated metabolic changes may further stimulate these synergistic or additive effects (Capparelli et al. 2012a,b,c). Finally, these effects may also be promoted by the transcriptional and translational networks occurring as a result of the TGF-β-induced EMT of cancer cells (Moustakas and Heldin 2014).

TGF-β signaling also triggers tumor angiogenesis. Cooperative actions with other signaling cascades in an autocrine/paracrine manner, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), PDGF, Notch, connective tissue growth factor (CTGF), and angiopoietin, stimulate angiogenesis by promoting endothelial cell migration and proliferation (ten Dijke and Arthur 2007; Sakurai and Kudo 2011; Neuzillet et al. 2014). Interestingly, depending on the TGF-β signaling levels and the TGF-β receptor status, a context-dependent angiogenic or antiangiogenic effect is observed. These threshold-dependent effects highlight the complex and intricate circuitry that defines how a given cell reads TGF-β signaling. Low levels of TGF-β signaling contribute to angiogenesis indirectly by inducing the expression of proangiogenic factors (VEGF, bFGF, CTGF) and other activities such as proteases, whereas high levels of signaling through Smad2 and Smad3 stimulate basement membrane formation, recruit smooth muscle cells, and inhibit endothelial cell growth (Sakurai and Kudo 2011). These effects are recapitulated in hepatocellular carcinoma and gliomas (Ito et al. 1995; Zhang et al. 2011).

TGF-β INDUCES EMT IN CANCER PROGRESSION

The role of TGF-β in promoting tumor progression and fibrosis has long been associated with

its capacity to produce an EMT through activation of E-cadherin repressors and EMT inducers (Acloque et al. 2009). EMT is crucial for normal development and is a pathological feature of cancer invasion (Thiery 2003; Lamouille et al. 2014). Cells undergoing EMT are characterized by a decrease or loss of E-cadherin expression and epithelial cell junctions, and an architectural rearrangement of the cytoskeleton into a mesenchymal pattern, resulting in cell motility and invasive properties. EMT is pivotal in various embryonic processes, such as gastrulation and formation of neural crest and structures of the heart. This process is driven by a set of transcription factors belonging to the zinc-finger, bHLH, and forkhead families, including Snail (Snail1), Slug (Snail2), Twist, and the zinc finger E-box binding homeobox proteins ZEB1 (also known as δEF1) and ZEB2 (also known as SIP1). By inducing EMT, TGF-β allows cancer cells to acquire the capacity to invade and disseminate.

In cancer, TGF-β-induced EMT occurs in transformed epithelial cells that are capable of propagating a tumor (Mani et al. 2008). In this context, the EMT program supports tumor invasion and dissemination by releasing tumor cells into the surrounding environment and promoting their motility. Tumor cells with EMT features have been shown to lead the cancer invasion fronts, and therefore these cells are probably the first to leave the primary tumor to then colonize distant sites and disseminate the disease (Friedl et al. 2012). Collectively, EMT confers invasiveness, motility, and progenitor-like features that are required for metastatic spread to this cell population. In addition, EMT also contributes to chemoresistance in breast and pancreatic cancer (Zheng et al. 2015). These features of EMT occur in a concerted manner under some circumstances; however, they are not always observed concomitantly, and individually do not imply cellular engagement in EMT. Although EMT is important for tumor dissemination, malignant cells commonly revert to an epithelial phenotype through a mesenchymal-to-epithelial transition (MET) that is required to colonize distant sites and form metastases (Polyak and Weinberg 2009; Ocana et al. 2012). Therefore, it is tempting to speculate that reduced exposure to or modulation of TGF-β signaling on dissemination contributes to such a phenotypic reversion. Consistent with this notion, TGF-β-induced expression of Id1 promotes metastatic colonization in breast cancer cells (Gupta et al. 2007) and represses Twist expression in basal breast cancer cells that infiltrate the lung parenchyma (Stankic et al. 2013). Although one may speculate that local high concentrations of the TGF-β may define TGF-β-mediated EMT before cell dissemination from the primary tumor, it is unknown why, when, or where MET is engaged and the extent to which TGF-β contributes to this process.

TGF-β was initially shown to induce an EMT in mammary cell lines and in mouse models of skin carcinogenesis (Miettinen et al. 1994; Portella et al. 1998; Thiery 2003; Derynck and Akhurst 2007). Mechanistically, the concerted action of TGF-β signaling, oncogenic Ras, and mutant p53 leads to p63 sequestration, causing inactivation of the tumor-suppressive function of the growth factor (Adorno et al. 2009). Under these circumstances, TGF-β and Ras cooperate to induce Snail1 expression and EMT in epithelial cells (Oft et al. 1996; Peinado et al. 2003; Derynck and Akhurst 2007). Accordingly, forced expression of Snail1 confers resistance to TGF-β-induced apoptosis and is sufficient to promote EMT in adult hepatocytes. In contrast, Snail1 depletion restores the cell death response (Franco et al. 2010). In mammary epithelial cells with increased Ras expression, TGF-β-induced EMT depends on NF-κB (Huber et al. 2004). In contrast, poly(ADP-ribose) polymerase-1 (PARP-1) dissociates Smad complexes from DNA, thereby attenuating TGF-β signaling and EMT (Lönn et al. 2010). In other contexts, Smad-mediated complexes may indirectly control the expression of *SNAIL1*, *SNAIL2*, and *TWIST* through high mobility group A2 (HMGA2) (Thuault et al. 2006). Additionally, TβRII-mediated phosphorylation of Par6 may also result in the resolution of cell junctions, thereby contributing to cell migration and invasion (Ozdamar et al. 2005). Generally, TGF-β-induced EMT is considered a protumorigenic

event. However, in TGF-β-sensitive pancreatic ductal adenocarcinoma (PDA) cells EMT becomes lethal by converting TGF-β-induced Sox4 from an enforcer of oncogenesis to a proapoptotic trigger (David et al. 2016). In these cells, Smad4 is necessary for EMT but not for Sox4 induction by TGF-β. Thus, the Smad4 inactivation status defines whether TGF-β can be protumorigenic or tumor-suppressive (David et al. 2016). Collectively, these results highlight a strong interdependence between TGF-β and EMT induction, and illustrate that the cellular outcome is subject to cell-type- and context-specific determinants.

In human cancers, the TGF-β-induced EMT gene program is encompassed within the gene expression profile of $CD44^{+}/CD24^{low}$ breast cancer cell population, which are seen as the tumor-initiating cells (Shipitsin et al. 2007), suggesting that activation of TGF-β signaling leads to EMT. Blockade of TβRI signaling, using specific kinase inhibitors, causes reexpression of epithelial-like characteristics. This implies that the $CD44^{+}/CD24^{low}$ tumor-propagating cell population may have undergone EMT, and that this process is, in part, mediated by TGF-β. The existence of tumor-initiating cells or so-called cancer stem cells (CSCs) is built on the concept of cellular plasticity, which is in agreement with the finding that TGF-β-induced EMT confers stem-cell-like properties (Mani et al. 2008), thereby linking the two concepts.

TGF-β also supports the generation of fibroblasts from epithelial cells through EMT, and from endothelial cells through a closely related process named endothelial-to-mesenchymal transition (EndMT). Adult fibroblasts are traditionally thought to propagate by proliferation (Weber 1997) or to differentiate from embryonic mesenchymal cells (Maric et al. 1997; Lang and Fekete 2001). However, during kidney, lung, liver, and tumors fibrosis, bone marrow-derived, endothelial, and epithelial cells contribute to fibroblast accumulation (Iwano et al. 2002; ten Dijke and Arthur 2007). As described above, TGF-β, in combination with other stimuli such as EGF and FGF-2 (bFGF), promotes EMT. Concomitant activation of Ras, Src, and other pathways facilitates important transcriptional regulation that leads to loss of adhesion and induction of the EMT. This process produces new fibroblasts in a model of experimental renal fibrosis under pathologic stress (Iwano et al. 2002).

EndMT plays a normal role in embryonic development of the heart, and has pathological roles in pulmonary fibrosis and in response to hypertension. The endocardium produces a mesenchymal cell population in the atrioventricular cushion, the primordia of the valves and the septa of the adult heart through EndMT (Eisenberg and Markwald 1995). This process occurs in a spatiotemporally restricted manner in the outflow tract and atrioventicular canal. It is triggered by TGF-β and BMP signals from the myocardium (Camenisch et al. 2002; Liebner et al. 2004), and leads to cardiac fibrosis and fibroelastosis when deregulated (Zeisberg et al. 2007b; Zeisberg and Kalluri 2015). In this context, TGF-β1 promotes cardiac fibrosis by inducing EndMT in adult coronary endothelial cells, whereas BMP-7 reverses this effect. In the lung, EndMT occurs in bleomycin-induced fibrosis (Hashimoto et al. 2010; Choi et al. 2016), thus providing a source of myofibroblasts. Similarly, EndMT is pivotal in the accumulation of mesenchymal-like cells in obstructive pulmonary vascular lesions that cause pulmonary hypertension (Ranchoux et al. 2015). Both processes are TGF-β-dependent and inhibited by the endothelial heat shock protein 1 (HSPB1) (Choi et al. 2016). Furthermore, EndMT relies on the EMT transcription factor Snail (van Meeteren and ten Dijke 2012). Outside the context of fibrosis, EndMT is an important source of cancer-associated fibroblasts in the Rip-Tag2 mouse model of pancreatic carcinoma, and myofibroblast accumulation has been established in solid tumors (Zeisberg et al. 2007a; Erez et al. 2010).

Tumor-Initiating Properties

Cancer cells endowed with tumor-initiating capacities, or CSCs, account for the generation of tumors at the primary site, as well as on dissemination (Oskarsson et al. 2014). Their properties are particularly significant in metastatic

cancer dissemination, when cells are subjected to highly adverse conditions and only few cells eventually succeed in generating secondary tumors. Cancer progression involves cell proliferation, invasion, migration, dissemination through circulation, extravasation and survival on arrival at distant sites, and eventual colonization. However, when cells do not have tumor-initiating properties, metastatic dissemination may not cause clinical symptoms. CSC properties may already be present in the primary tumor. Cell heterogeneity is characteristic of many cancers, as a result of hierarchical organization or exposure to environmental cues, and cancer cells expressing such markers are present in patients and capable of generating metastases when inoculated into immunodeficient mice (Pece et al. 2010; Merlos-Suárez et al. 2011; Baccelli et al. 2013).

TGF-β enhances the CSC potential in glioblastoma (Anido et al. 2010) and collaborates with canonical and noncanonical Wnt signaling to induce activation of mesenchymal CSC traits in association with an EMT program (Scheel et al. 2011). TGF-β sustains a $CD44^{high}/Id1^{high}$ glioma-initiating cellular population responsible for tumor initiation, relapse, and therapeutic resistance (Anido et al. 2010). In addition, TGF-β also supports self-renewal of glioma-initiating cells through the induction of *Sox2* expression (Ikushima et al. 2009) or the expression of leukemia inhibitory factor (LIF) (Peñuelas et al. 2009). LIF is a cytokine with a crucial role in embryonic stem cells. It is highly secreted by cells in certain glioblastomas, promotes self-renewal of cancer-initiating cells and facilitates tumor relapse (Peñuelas et al. 2009). This cell population tends to be localized at the perivascular niche and edges of tumors, and confers poor prognosis in glioblastoma patients. TGF-β has also been shown to induce the CSC marker CD133 in hepatic epithelial cells that behave aggressively when grafted in mice (You et al. 2010) and gain resistance to chemotherapy and TGF-β-mediated apoptosis (Ding et al. 2009). A critical role for TGF-β in maintaining leukemia-initiating cells has also been proposed in chronic myeloid leukemia (CML). In these cells, TGF-β induces Akt activation, and controls FoxO3a localization and inactivation, and its inhibition concomitantly with that of a BCR-ABL causes an efficient depletion of CML in vivo (Naka et al. 2010). In-depth studies suggest that the TGF-β–FoxO–BCL6 axis interacts with nutrient signaling to maintain CML stem cells. This signal integration relies on FoxO3–Smad3 association and is controlled by p38 MAPK activity (Naka et al. 2015). Overall, the evidence that TGF-β promotes the generation and maintenance of CSC features is compelling.

As described already, TGF-β-induced EMT and tumor-initiating properties occur concomitantly at times (Valastyan and Weinberg 2011), and both the EMT and stem-cell-like markers are coexpressed in circulating tumor cells from patients with metastasis (Aktas et al. 2009; Baccelli et al. 2013; Yu et al. 2013). Although TGF-β induces EMT, and thus promotes loss of adhesion and polarity of malignant cells at the invasive front and acquisition of migration properties (Oft et al. 1996; Xu et al. 2009), enforced expression of EMT transcription factors, such as Twist in breast cancer cells provides stem-cell assets (Mani et al. 2008; Wellner et al. 2009; Scheel et al. 2011). In addition to the role of TGF-β signaling in EMT and, hence, in stem-cell-like properties, other TGF-β family members play key roles in cancer progression and metastasis by suppressing self-renewal and promoting cancer cell differentiation. BMP signaling in the lung parenchyma or bone cavity was shown to impose latency in breast cancer cells by restraining CSC properties and supporting the differentiation of malignant cells, as well as by modulating the metastatic site at the bone. Coco or related BMP-sequestering antagonists support lung metastatic progression (Gao et al. 2012), whereas Noggin reinforces bone colonization by breast cancer cells in a cell-autonomous and nonautonomous manner (Tarragona et al. 2012).

TGF-β in Metastasis

The function of TGF-β in cancer progression extends beyond the primary site and has also been implicated in facilitating distant metastasis. The expression of TGF-β1 in infiltrating

breast carcinomas has long been associated with metastatic outcomes (Dalal et al. 1993), whereas low expression of TGF-β receptors in ER-negative breast cancers has been linked to favorable prognosis (Buck et al. 2004). In addition, blockade of TGF-β signaling in mice with mammary tumors that were subjected to radiation or chemotherapy has been shown to prevent lung metastasis (Biswas et al. 2007). Taken together, the results of many different studies implicate TGF-β signaling in metastatic dissemination. However, contradictory observations have been reported in mouse models. Whereas the expression of activated TGF-β1 in *ErbB2/Neu* mouse mammary tumors enhances metastasis (Muraoka et al. 2003), expression of a dominant-negative TβRII unexpectedly also promotes metastasis in the same model (Novitskiy et al. 2014). TβRII depletion by targeted gene inactivation or dominant-negative interference increases metastasis in polyoma middle-T antigen (PyMT)-tumors (Forrester et al. 2005), and inhibits metastasis of prostate cancer xenografted in mouse (Zhang et al. 2005a). Thus, contextual cues based on tumor type or event within tumor subtypes appear to have a pivotal effect on the potential of TGF-β signaling to trigger a metastatic outcome.

In ER-negative breast cancer, TGF-β signaling has been associated with lung metastasis (Padua et al. 2008). Transient exposure of breast cancer cells to TGF-β promotes their extravasation from blood vessels and entry into the lung by activating the expression of the adipokine ANGPTL4 (Padua et al. 2008). That study revealed that the later stages of metastasis are influenced by transient signals produced in the primary tumor microenvironment. Similarly, TGF-β- and Smad-dependent induction of *PTHLH*, which encodes parathyroid hormone-like protein (also known as PTHrP), *CTGF*, and *JAGGED1* may enhance osteolytic metastasis of breast, prostate and melanoma cancer cells (Kang et al. 2003b; Mohammad et al. 2011; Sethi et al. 2011; Xu et al. 2015) and has been confirmed in malignant cells derived from metastatic breast cancer (Gomis et al. 2006b). In prostate cancer, TGF-β also significantly upregulates *PMEPA1* expression. PEMPA1 interacts with R-Smads and ubiquitin ligases, blocking TGF-β signaling independently of the proteasome (Fournier et al. 2015). Blockade of this negative feedback loop by methylation of the *PMEPA1* promoter increases the prometastatic features of prostate cancer (Fournier et al. 2015). The development and outgrowth of bone metastatic lesions relies on a cellular and molecular network of interactions between cancer and stromal cells of the bone microenvironment, a process in which TGF-β plays a pivotal role. Malignant breast cells have the capacity to alter the balance between the two main bone-preserving cell populations, namely, osteoblasts and osteoclasts, leading to bone destruction and metastatic growth. PTHrP produced by tumor cells promotes osteoclast differentiation, resulting in bone destruction, which in turn leads to increased availability of growth factors, including TGF-β, which are stored in bone matrix, thus further stimulating the malignancy of the transformed cells in what is named a "vicious cycle" (Guise et al. 1996; Mundy 2002). TGF-β released on osteoclast degradation of the bone causes a signal in tumor cells, thus triggering Smad-dependent Jagged1 expression, which in turn further supports the osteoblast differentiation induced by Notch (Sethi et al. 2011). Concomitantly, pathological TGF-β release from the bone causes muscle weakness by decreasing Ca^{2+}-induced muscle force. TGF-β-induced Smad signaling drives the expression of NADPH oxidase 4 (Nox4) in the muscle, which in turn increases ROS production and intracellular Ca^{2+} depletion (Waning et al. 2015). Overall, the contribution of TGF-β signaling to metastasis is incompletely understood, and appears to cover a broad spectrum of tumor types and pathological consequences. Further research efforts are needed to gain a more comprehensive perspective.

CONCLUSIONS AND THERAPEUTIC IMPLICATIONS

From the study of the pathways that govern tumorigenesis and cancer progression, numerous novel putative therapeutic targets have emerged, among them the TGF-β pathway. Sev-

eral small and large molecule compounds have been developed with the aim to inhibit TGF-β signaling. However, TGF-β exerts dual and opposing roles in oncogenesis. Therefore, a detailed understanding of the TGF-β biology in cancer is required to design successful therapeutic approaches and prevent unwanted side effects. The dual effect of TGF-β on cancer is explained by the pleiotropic nature of TGF-β. Gene responses to TGF-β are determined by the cellular context and the integration of the TGF-β pathway with other signals received by the cell. Such an understanding of the cellular context that determines the switch of the TGF-β pathway toward a tumor-promoting factor is essential to predict in which patients TGF-β signaling might be a suitable therapeutic target. Little by little, we are acquiring greater knowledge of the TGF-β pathway. This knowledge will ultimately allow the rational design of several clinical trials to test anti-TGF-β compounds. However, to fully evaluate the complex and pleiotropic TGF-β pathway as a valid therapeutic target against cancer, further research is required to unravel the molecular mechanisms involved in the loss of the tumor-suppression response and gain of the oncogenic response to this cytokine.

ACKNOWLEDGMENTS

We thank J. Urosevic and V. Chiganças for helpful discussions and I. Joval for assistance in mounting the figures. J.S. and R.R.G. are supported by the Institució Catalana de Recerca i Estudis Avançats. Their research is supported by Grants from the Generalitat de Catalunya (2014 SGR 535) and the Spanish Ministerio de Ciencia e Innovación (MICINN) (SAF2013-46196, FEDER funds) to R.R.G., and from the Asociación Española contra el Cáncer (AECC), the Josef Steiner Foundation, and FIS PI13/02661 to J.S.

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Cold Spring Harb Perspect Biol published online February 28, 2017

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